HHS Public Access

Author manuscript

Microchem J. Author manuscript; available in PMC 2024 October 01.

Published in final edited form as:

Microchem J. 2023 October ; 193: . doi:10.1016/j.microc.2023.109088.

A Portable, Encapsulated Microbial Whole-Cell Biosensing System for the Detection of Bioavailable Copper (II) in Soil

Md Harun Or Roshid[a,b,c], **Michael Moraskie**[b,c], **Gregory O'Connor**[b,c], **Emre Dikici**[b,c], **Jean-Marc Zingg**[b,c], **Sapna Deo**[b,c], **Leonidas G. Bachas**[a,c,*], **Sylvia Daunert**[b,c,d,e,*]

[a.]Department of Chemistry, University of Miami, Miami, FL 33146

[b.]Department of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, Miami, FL 33136

[c.]The Dr. John T. Macdonald Foundation Biomedical Nanotechnology Institute - BioNIUM, University of Miami, Miami, FL 33136

[d.]The Miami Clinical and Translational Science Institute, University of Miami, Miami, FL 33146

[e.]Sylvester Comprehensive Cancer Center, University of Miami, Miami, FL 33146

Abstract

A portable, field deployable whole-cell biosensor was developed that can withstand the complex matrices of soil and requires minimal to no sample preparation to monitor bioavailable concentrations of the essential micronutrient copper (II). Conventional measurement of micronutrients is often complex, laboratory-based, and not suitable for monitoring their bioavailable concentration. To address this need, we developed a fluorescence based microbial whole-cell biosensing (MWCB) system encoding for a Cu^{2+}-responsive protein capable of generating a signal upon binding to Cu^{2+}. The sensing-reporting protein was designed by performing circular permutation on the green fluorescent protein (GFP) followed by insertion of a Cu^{2+} binding motif into the structure of GFP. The design included insertion of several binding motifs and creating plasmids that encoded the corresponding sensing proteins. The signal generated by the sensing-reporting protein is directly proportional to the concentration of Cu^{2+} in the sample. Evaluation of the resulting biosensing systems carrying these plasmids was performed prior to selection of the optimal fluorescence emitting Cu^{2+}-binding protein. The resulting optimized biosensing system was encapsulated in polyacrylate-alginate beads and embedded in soil for detection of the analyte. Once exposed to the soil, the beads were interrogated to measure the fluorescence signal emitted by the sensing-reporting protein using a portable imaging device.

[*]Corresponding Author Information Leonidas G. Bachas, bachas@miami.edu, Sylvia Daunert, sdaunert@med.miami.edu.

Author contributions. M. H. O. Roshid, M. Moraskie, G. O'Connor, E. Dikici, J.M. Zingg, S. Deo, L. G. Bachas and S. Daunert contributed to study design and concept. M. H. O. Roshid performed experiments and acquired data. M. H. O. Roshid analyzed data and wrote the manuscript. M. Moraskie, G. O'Connor, E. Dikici, J.M. Zingg, S. Deo, L. G. Bachas and S. Daunert contributed to manuscript revisions. All authors approved of the final version to be published.

Competing interests: The authors declare no competing interests.

The biosensor was optimized for detection of Cu^{2+} in terms of selectivity, sensitivity, matrix effects, detection limits, and reproducibility in both liquid and soil matrices. The limit of detection (LoD) of the optimized encapsulated biosensor was calculated as 0.27 mg/L and 1.26 mg/kg of Cu^{2+} for Cu^{2+} in solution and soil, respectively. Validation of the portable imaging tools as a potential biosensing device in the field was performed.

Keywords

Whole cell biosensors; Engineered sensing-reporting protein; Environmental monitoring; Copper (II) biosensing; Bioavailable copper; Portable biosensing system; Biocontainment; Polyacrylate-alginate beads

1. Introduction

Improving food security necessitates an increase in food output either by an expansion of agricultural land or an increase in the frequency of land cultivation; given the former option is limited, the latter can be achieved by sustainable agriculture and soil management (FAO et al. 2021; Graham et al. 2001; Sadowski and Baer-Nawrocka 2018; WPP 2019). One of the ways forward is to maximize output via optimization of agricultural conditions such as macro and micronutrients, pH, organic and inorganic content, and moisture (Fageria et al. 2002; Fernández and Hoeft 2009; Sattari et al. 2014). To successfully optimize these parameters, active monitoring of the soil and the conditions is necessary (Karlen et al. 2019; Lehmann et al. 2020). There are a number of commercially available artificial intelligence-based remote sensing services available to monitor environment and soil health, including monitoring the weather, crop sustainability, weed detection, diseases and pests, fighting mildew, as well as irrigation levels (Jung et al. 2021). For the purposes of active monitoring, farmers need rapid, portable, and on-site sensors that are robust, and easy to use. Integrating the field data gathered from these sensors, combined with artificial intelligence and mathematical algorithms that predict possible outcomes, will help farmers make informed decisions regarding soil management. Although current sensors provide plenty of information for proper soil management, there is still a need for sensors that provide data specifically on the availability of nutrients in the soil. Therefore, an innovative, portable sensing system capable of measuring bioavailable micronutrients will ensure proper soil management for maximum crop yield.

Copper is an essential micronutrient for living organisms (Alloway 2013), and is of particular importance for plant growth, development, and seed production, as evident by its involvement in respiration, photosynthesis, carbohydrate distribution, and protein metabolism (Cohu and Pilon 2010; Kumar et al. 2021; Pessarakli et al. 2015; Rehman et al. 2019). The natural total abundance of copper in soil varies depending on the geography and type of soil and is typically between 2 and 250 mg/kg of soil (ATSDR 2004). However, not all copper in the soil can be readily absorbed by plants because it may be sequestered by organic matter (Kumar et al. 2021; Rehman et al. 2019). A concentration of 5 to 30 mg of copper per kg of crop tissue is considered sufficient for the majority of plant species (Ballabio et al. 2018). Therefore, an important parameter to consider when evaluating copper

as a micronutrient is the amount of bioavailable copper present, which is the fraction of the metal that is in a form that organisms can uptake for their cellular activities (Adrees et al. 2015; Kim et al. 2015; Kumar et al. 2021). Conventional Cu^{2+} analysis of agricultural soils typically relies on chemical extraction methods in combination with highly precise instrumentation that tends to overestimate the levels of bioavailable Cu^{2+} (Arenas-Lago et al. 2014; Chaignon et al. 2003; García-Marco et al. 2020; Nogueirol et al. 2010; Provenzano et al. 2010). Furthermore, their use as in-field analytical tools is severely limited due to their complicated setup, high maintenance, and operational costs. The need for portable, ready-to-use analytical tools for the in-field rapid detection of bioavailable micronutrients has galvanized the development of a variety of alternative analytical tools, such as microbial whole-cell biosensors (MWCB) (Moraskie et al. 2021). Herein, we propose a MWCB that can supplant conventional analytical approaches and be employed for the routine monitoring of bioavailable Cu^{2+} ions in the field.

Many previously reported MWCBs for copper detection rely on the active transcription and translation of reporter proteins as signals which, despite having satisfactory analytical parameters, are severely limited for field application i.e. direct soil analysis due to their need for optimal nutritional and growth conditions (pH, temperature) for proper functionality and reproducibility (Chen et al. 2017; Costello et al. 2020; Kang et al. 2018; Li et al. 2014; Moraskie et al. 2021). Given that field conditions are highly variable depending on a variety of factors such 94 as weather, temperature, pH and soil composition, these MWCBs cannot be used directly for soil monitoring. Further complicating the widespread use of these MWCBs are regulatory concerns regarding the release of genetically modified organisms and their potential impact on the natural ecology (Hollander 2018; Snow et al. 2005). As a result, several biocontainment strategies have been proposed to address this issue, including the use of synthetic gene circuits and kill switches as well as physical biocontainment methods such as hydrogels. To develop field-deployable MWCBs, encapsulation can be a key component in minimizing microorganism release to the environment (Lee et al. 2018; Tang et al. 2021). However, the main concern regarding encapsulation of microorganisms is that their optimal performance is hindered due to an inability to supply growth media. In order to overcome these disadvantages, we propose a MWCB which does not require growth media to perform optimally and can, therefore, function properly while encased in a polymer matrix. Encapsulation of our MWCBS not only protects the organisms from harsh environments, but it also conveniently acts as an optimal vessel for the effortless embedding and removal of the sensors from soil samples and their subsequent measurement in a portable device. The novelty of our approach lies in the incorporation of a self-reporting sensing protein whose fluorescent signal changes in the presence of copper in a whole cell bacterium.

It is well known that copper (II) can bind to a wide variety of proteins (Cohu and Pilon 2010; Inesi 2017); and through analysis of these copper-binding proteins, researchers identified the amino acid sequences to which Cu^{2+} binds (Frączyk 2021; Schwab et al. 2016). Incorporating these Cu^{2+} sequences into proteins and peptides has led to the development of several biosensors for copper ions (Forzani et al. 2005; Synhaivska et al. 2019; Yang et al. 2003). For example, by inserting “Amino Terminal Copper (II) and Nickel (II)” or ATCUN motif at the amino termini of the circularly permutated green fluorescent

protein, Choi et al. developed a protein-based biosensor for copper (Choi et al. 2015). However, the developed protein-based biosensor, while highly sensitive, requires several protein purification steps and lacks the field-deployability and direct application capabilities of MWCBs. A comprehensive list of proteins containing an ATCUN-like motif can be found elsewhere (Frączyk 2021; Maiti et al. 2020; Sankararamakrishnan et al. 2005). The ATCUN motif can be constructed by combining amino acids in the order Xaa-Zaa-His, with Xaa being an amino acid with a free N-terminal amine, Zaa being any amino acid except proline, and histidine (His) (Fraczyk 2021). In the study described in this manuscript, a total of eight different biosensors were developed for Cu^{2+} by inserting such Cu^{2+}-binding amino acid sequences (ATCUN motif) in the newly formed N-terminus of a circularly permutated green fluorescent protein (cpEGFP) (Supplementary Figure S1 & supplementary Table S1-S2). Our approach resulted in a genetically modified fluorescent protein that can selectively bind copper ions, is expressed inside a whole cell bacterium, and can be employed to detect Cu^{2+} directly in soil without any further protein purification. A soil-borne spore-forming bacterium, *Bacillus subtilis*, was used to express this engineered fluorescent protein and sense the bioavailable copper. We employed *Bacillus subtilis* for our work since it is a plant-growth promoting rhizobacteria used in sustainable agriculture (Mahapatra et al. 2022), and is generally recognized as safe (GRAS) organism (Harwood 1992). The performance of the *B. subtilis* MWCB carrying copper-binding cpEGFP was evaluated in terms of fluorescence, stability, detection limit, reproducibility, selectivity, and storage conditions. Expression of the self-reporting sensing protein and subsequent encapsulation of the cells prior to deployment enables the selective, sensitive, and rapid detection of bioavailable copper in soil samples, performed on-site via a portable imaging device, without the need for nutrient supplementation or pretreatment of the samples (Figure 1). Additionally, this MWCB can be prepared and stored at room temperature prior to use.

2. Materials and methods

2.1 Reagents and bacterial strains

Chemicals such as copper (II) sulfate pentahydrate, sodium chloride, potassium chloride, magnesium chloride, iron (III) chloride, zinc chloride, calcium chloride, manganese (II) sulfate, nickel (II) sulfate, lead (II) chloride, iron (II) sulfate, iron (III) chloride, aluminum (III) nitrite, sodium alginate, agar, antibiotics (ampicillin and chloramphenicol), clean loam soil (certified reference material, trace metal free), clean clay soil (certified reference material, trace metal free), metals in soil (certified reference material, concentration of different metals is known) standard, PCR grade water, growth media 2xYT and nutrient broths were purchased from MilliporeSigma (St. Louis, MO). Cloning strain *E. coli* NEB® 5-alpha, restriction enzyme sets (*Bam*HI-HF & *Xma*I), and Q5 hot start master mix were purchased from New England Biolabs (Ipswich, MA). Shuttle vector pHT2133 and *Bacillus subtilis* 168M were purchased from MoBiTec (Germany). Everglades clay soil and all-purpose garden soil were collected locally. The Invitrogen E-gel electrophoresis system was purchased from ThermoFisher Scientific.

2.2 Plasmid construction

The gene for enhanced green fluorescent protein (EGFP) was cloned from previously constructed plasmid pSD202 (Date et al. 2010). To obtain a protein in which the chromophore is more exposed, a strategy was pursued through which the N- and C-termini of EGFP become linked and new N- and C-termini are generated (Choi et al. 2015). This required cutting the original EGFP into two fragments and reconnecting them with a flexible linker. Accordingly, in the first round of cloning, four sets of primers were used to amplify and rejoin the two *egfp* fragments using a flexible linker GGTGGS by overlap extension polymerase chain reaction. The primers also contained sequences for the restriction enzymes, *Bam*HI and *Xma*I. The resulting circularly permutated *egfp* (cpEGFP) was then cloned under a constitutive expression system in pHT2133 (MoBiTec) plasmid, using two restriction enzyme sets *Bam*HI-HF and *Xma*I, to facilitate the expression of stable and high levels of the protein. Plasmid pHT2133 has a *Pgrac* constitutive promoter system that expresses high levels of target protein without the addition of exogenous inducer. This newly constructed plasmid was then transformed into *E. coli* NEB® 5-alpha cells (New England Biolabs) and verified by Sanger sequencing and named as pSDM1. As the amino acid sequence of copper binding ATCUN motif can be in the order of Xaa-Zaa-His, with Xaa being an amino acid with a free N-terminal amine, Zaa being any amino acid except proline, and histidine (His), a series of site directed mutagenesis experiments were performed to this newly constructed plasmid, pSDM1, using a set of primers to introduce the desired amino acid mutations in the newly formed N-terminus of the cpEGFP. A total of eight different ATCUN motifs were constructed. A detailed cloning strategy, including amino acid sequences of the constructed sensing-reporting protein, can be found in the attached supplementary material. After verifying the sequences of the constructed plasmids, named as pSDM1–8, the plasmids were used to transform *Bacillus subtilis* strain 168M following the transformation protocol of the supplier. *Bacillus subtilis* strain 168M carrying plasmids pSDM1–8 was named as biosensor BSD1–8. A list of primers, strains, as well as constructed biosensors in this experiment is provided in Supplementary Table S1 & S2.

2.3 Bacterial growth

As the constructed whole cell biosensing systems were constitutively expressed, the growth conditions of these biosensors were relatively simple. For the screening of fluorescence: a 5.0 mL overnight culture of nutrient-rich 2xYT medium (16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl) containing 5.0 μg/mL chloramphenicol was inoculated with the specific biosensor, i.e., BSD1–8, and was grown at room temperature. The following day, bacterial cells were harvested, washed, and resuspended in deionized water. A fluorescence emission spectrum was obtained by using a microtiter plate reader (CLARIOstar Plus, BMG Labtech, Cary, NC). The excitation wavelength was set at 462–16 nm, where the center wavelength (CWL) was at 462 nm, and the full width at half-maximum (FWHM) was 16 nm; the emission spectrum was recorded between 490 nm and 610 nm. After the initial fluorescence screening, biosensors version BSD1/4/5/6 were not further considered for exhibiting low to no fluorescence emission, and BSD2/3/7/8 were grown according to the following optimized protocol: A 5.0-mL overnight culture of 2xYT medium containing 5.0 μg/mL chloramphenicol was inoculated with a biosensor, i.e., BSD2/3/7/8, and was grown at 37 °C. The following day, a large volume, usually 50.0 mL of 2xYT, was inoculated from

the overnight culture to give a final 1:100-fold dilution and was grown at 37 °C for 2 h, and then transferred to a 22 °C incubator for overnight incubation at 200 rpm. The following day, bacterial cells were harvested and washed four times using deionized water. Finally, the optical density of the bacterial culture in deionized water was measured at 600 nm and adjusted to ~1.0.

2.4 Standard preparation and assay protocol in the liquid culture

Copper standards ranging from 0.005 to 250 mg/L were prepared by dissolving copper (II) sulfate pentahydrate in distilled water at a concentration of 1×10^{-2} M (635 mg/L) of Cu^{2+}, and then preparing serial dilutions from this stock solution in distilled water. Standards for other metals were prepared in the same manner. Standard assays were performed at room temperature and in triplicate using 90 μL of whole-cell suspension and 10 μL of standards.

2.5 Fluorescence Detection

The fluorescence assay was performed by using 90 μL of whole cell biosensors, i.e., 208 BSD2/3/7/8, and 10 μL of 1 mg/L copper standard (or 10 μL of deionized water as blank) in triplicate. The assay mixture was immediately transferred to a microtiter plate reader instrument (BMG, Labtech), and the change in fluorescence intensity was recorded for 150 min at 5 min interval and 200 rpm double orbital shaking. Excitation and emission wavelengths were set between 462–16 nm and 510–15 nm, respectively. Then, the obtained intensity change data were normalized against the corresponding blank data at the same time point to minimize potential 214 photobleaching and expressed as percentage.

2.6 Selectivity Studies

A volume of 90 μL of whole cell biosensor and 10 μL of metal standard solution (or 10 μL of deionized water as blank) were mixed and incubated at 200 rpm for 60 min, after which the change in fluorescence intensity was measured using a microtiter plate reader.

2.7 Whole cell biosensor (BSD8) encapsulation in Polyacrylate alginate beads and soil studies

An amount of 1.0 g clean loam soil per well was weighed and spiked with varying Cu^{2+} standards ranging from 0.005 to 250.0 mg/L in a 24-well Greiner Bio-One microtiter plate by pipetting 1.0 mL of standards. Spiked soil samples were lyophilized and stored in a dry condition. For the bacterial culture, a large 300 mL 2xYT medium containing 5 μg/mL chloramphenicol was inoculated from a 3.0-mL overnight startup culture of biosensor BSD8 to give a 1:100-fold dilution and was grown overnight at room temperature. The following day, bacterial pellets were collected and resuspended in deionized water and centrifuged at 6000xg to collect the washed bacteria; this process was repeated three additional times. Bacterial whole cells were encapsulated in polyacrylate-alginate beads. For the encapsulation in polyacrylate-alginate beads, 1.0 g of sodium alginate was measured and dissolved completely in 45 mL deionized water after vigorous overnight stirring. The following day, 5 mL of 10% polyacrylic acid solution and 1.0 g wet-weighed whole cell pellets were added to 45 mL alginate solution, and the pH was adjusted to ~7.4 with 10.0 M NaOH. Polyacrylate-alginate beads were prepared by loading this suspension in a dropping

funnel (internal diameter 3.9 ± 0.01 mm) and allowed to drip into 0.2 M calcium (II) chloride solution by gravitational force. Freshly made beads were allowed to stay in the 0.2 M calcium (II) chloride solution for another hour and then washed twice using 0.9% sodium chloride solution supplemented with 0.1% Tween 80. Subsequently the beads were drained and placed in a 50-mL centrifuge tube and stored at room temperature and at 4 °C. The average diameter and weight of the encapsulated BSD8 beads were measured as 4.7±0.2 mm and 49.6±2.9 mg, respectively (n = 15 beads). The prepared polyacrylate-alginate beads were used to measure the copper (II) ions in copper standard solutions and copper spiked soil. To ensure that the copper ions are available to interact with the embedded sensing beads, a volume of 0.3 mL deionized water was added into the 24-well plates containing the spiked lyophilized soil and seven alginate beads, and further incubated for 5 h at room temperature without shaking. Then, the alginate beads were collected from the plate wells, washed with deionized water, and transferred into a clean microtiter plate after which the fluorescence intensity of each well was measured with the plate reader by exciting at 462–16 nm, while emission was recorded at 510–15 nm. Subsequently, the beads were placed on top of the E-Gel™ illuminator, and the images were taken using the E-Gel™ Power Snap camera (purchased from Invitrogen). The Invitrogen E-Gel Power Snap electrophoresis system consists of two components: a gel electrophoresis device and a snap camera. The electrophoresis unit utilizes blue LED to illuminate the gel at a center wavelength (CWL) of 465 nm, and full-width at half-maximum (FWHM) of 20 nm, which also serves as the excitation wavelength for the green fluorescent protein; the polyacrylate-alginate (PAA) encapsulated beads can be visualized in the illuminator.

2.8 Fluorescence stability of encapsulated whole cell biosensor BSD8

The stability of polyacrylate-alginate (PAA) encapsulated whole cell biosensor beads stored at room temperature and at 4 °C was confirmed by measuring the fluorescence intensity of the encapsulated beads on different days. To validate the viability of the encapsulated biosensor system for long term storage, a comparative assay of 50 mg/kg copper spiked soil and a non-spiked soil using both room temperature and 4 °C stored PAA beads were performed at day-0, day-5, day-10, day-18, day-26, and day-31.

2.9 Data analysis: Standard curve, Limit of Detection, and ImageJ processing

Data analysis of the MWCB was performed using GraphPad Prism 9 (version 9.4.0). The limit of detection was interpolated from the raw data fitted with an inhibitor vs response (variable slope, four parameters) non-linear curve using the three standard deviation subtracted average blanked value ($\mu B\text{-}3s.d._B$); μB is the average signal of triplicate blank, and $s.d._B$ is the standard deviation of the blank. Images of polyacrylate-alginate beads extracted from both copper standard solution and spiked soil were processed using ImageJ software. For comparison, a microtiter plate reader was also used to measure the change in fluorescence intensity at the completion of each assay. The signal in relative light units (RLU) of each polyacrylate-alginate bead was measured by using a circular selection that was exactly same in size for every bead in an image. Measured data were processed in the same manner mentioned above using the GraphPad Prism 9 software.

3. Results & Discussion

3.1. Screening biosensor constructs for Cu^{2+} detection

Since the folding temperature and maturation time of circularly permutated green fluorescent proteins are different from that of the wild type or non-circularly permutated version (Kostyuk et al. 2019), we evaluated these conditions rigorously. Generally, green fluorescent protein is a robust and stable fluorescent protein. Performing a circular permutation to green fluorescent protein opens the chromophore towards environmental insult, and can increase the folding time, reduce stability, and decrease the overall fluorescence intensity. Despite these shortcomings, circular permutation is an optimal way to bring a ligand, such as copper ions, close to the chromophore unit, so it can alter the fluorescence signal more efficiently, thereby increasing the signal-to-noise ratio and overall sensitivity of the assay.

Four separate studies were performed for the initial screening. In one study, a fluorescence emission scan was performed using an aliquot of 200 μL of the whole cell biosensors (BSD1/2/3/4/5/6/7/8) in triplicate. Biosensor constructs BSD1/4/5/6 yielded very low to no fluorescence in comparison to BSD2/3/7/8 and were, therefore, not further considered for the rest of the studies. Figure 2a represents the fluorescence intensity of BSD2/3/7/8 normalized to the optical density of the solution at 600 nm to account for potential differences in the number of cells present in each microtiter plate well. *Bacillus subtilis* whole cell biosensor BSD8 exhibited two-fold higher fluorescence than BSD3, and BSD7, and more than four-fold higher fluorescence than BSD2 (Figure 2a). As the fluorescence of the cpEGFP depends on the maturation and thermal stability of the fluorescent protein, variability was expected between these systems. Biosensors BSD2 and BSD3 only fluoresced when incubated at room temperature or below (between 16 to 23°C), while in comparison whole cell biosensors BSD7 and BSD8 demonstrated higher thermal stability and fluoresced even when incubated 10 °C above room temperature.

A time course fluorescence study was performed using biosensor constructs BSD2/3/7/8 in presence of 1.0 mg/L of Cu^{2+} standard to determine the comparative profile of the biosensor systems at the lower range of bioavailable copper in soil (Figure 2b). All tested biosensor systems demonstrated around 24–27% change in fluorescence intensity after 60 min except BSD8, which 301 showed around 12%.

The ATCUN motif can bind both copper (II) and nickel (II); therefore, a response comparison using soil-relevant concentrations of Cu^{2+} and Ni^{2+} was performed (Figure 2c). Figure 3c shows much higher change in fluorescence intensity than that of Figure 3b because of the difference in metal concentrations, i.e., 25–50 mg/kg vs 1 mg/kg of copper. A concentration of 25 mg/L of nickel was chosen to investigate how much change is observed when nickel concentrations are similar to copper concentrations. However, nickel ions occur at lower concentrations than copper ions in nature, therefore, we also compared the effect of nickel ions on the fluorescence at more relevant soil concentrations. A concentration of 13 mg/L of nickel was tested since this concentration is the average total nickel concentration in soils in the United States. Biosensing systems BSD2 and BSD8 demonstrated higher specificity towards Cu^{2+}. Finally, in another experiment, a copper standard assay was

performed using all four biosensors (Figure 2d). Based on these initial screening results, the biosensing system, BSD8 was chosen for further optimization because of its higher fluorescence intensity and higher specificity towards Cu^{2+} ions. A time course fluorescence assay using 1 to 100 mg/L of Cu^{2+} standard and BSD8 is shown in Figure 2e.

3.2. Characterization of *Bacillus subtilis* whole cell biosensing system harboring plasmid pSDM8 in liquid culture

Several calibration curves for copper (II) were generated at different days within a 12-month period to demonstrate the ruggedness of the biosensor system, BSD8 (Figure 3a). All assays were performed in triplicate. As it is evident from Figure 3a, the selected MWCB performed robustly and reproducibly. A limit of detection of 0.32 ± 0.08 mg/L was calculated for MWCB, BSD8 (Figure 3a) with a 95% confidence level.

Given the soil relevant concentration of copper is around 50 mg/L, a selectivity assay was performed using a variety of metals commonly found in soil at this copper relevant concentration, unless stated otherwise (Figure 3b) (Henckens and Worrell 2020). The term “Mix” refers to the mixture of all assayed metals at a concentration of 50 mg/L; and without copper or with copper. As it is evident from the selectivity studies, the biosensor responds strongly to copper. Notwithstanding, high concentrations of cobalt and nickel (50 mg/L) appear to change the fluorescence of the constructed biosensor system BSD8, albeit not as strongly as copper. However, according to the Agency for Toxic Substances and Disease Registry (ASTDR) report, the average total cobalt and nickel concentrations in US soil is 7.2 and 13.0 mg/kg, respectively (ATSDR 2004, 2005, 2022, 2023). As such, cobalt and nickel were further tested at their soil relevant concentrations, and we show that their interference at these relevant concentrations was significantly lower.

3.3 Copper (II) measurement in spiked soil using whole cell polyacrylate-alginate beads

One of the major concerns of in-field MWCBs deployment is the leaking of genetically modified microorganisms into the environment. To address this concern, several biocontainment strategies have been suggested including the use of synthetic gene circuits or kill switches, and physical biocontainment such as within hydrogels (Lee et al. 2018; Tang et al. 2021). Recently, Luisi et al. developed eBEADS, a polyacrylamide-alginate encapsulated genetically modified biosensing system to detect 2-phenylphenol in environmental samples without the concern of releasing the microbes in the environment (Luisi et al. 2022). Moya-Ramirez et al. also developed a living analytics in a multilayer polymer shell (LAMPS) to encapsulate bacterial biosensors and cocultured them with mammalian cells without the concern of releasing and disturbing the mammalian cell physiology (Moya-Ramírez et al. 2022). Herein, we have designed a method to deploy MWCB directly into the soil by encapsulating the MWCB in polyacrylate-alginate beads that work as physical biocontainment system and are easy to transport and use in the field.

In the developed MWCB assay, soil matrix effects were successfully mitigated as the beads can be isolated from soil, washed, and read in a portable instrument. The resulting images produced a clear distinction between varying copper (II) concentrations. The designed MWCB has potential as a rugged, portable, easy to use analytical tool for in-field monitoring

of bioavailable Cu^{2+} in soil. This strategy can be applied to other micronutrients of interest for monitoring their bioavailable levels in soil.

To facilitate the use of the biosensor system for detecting Cu^{2+} in soil, the BSD8 cells were encapsulated into the polymer matrix of polyacrylate-alginate beads. Whole cell-encapsulated polyacrylate-alginate beads are practical because of their high resistance to mechanical breakdown and their ability to be stored for long periods of time. We optimized the protocol for the incorporation of whole cells into the polymer matrix by evaluating various conditions, such as the percentage of the crosslinker, size of the alginate beads, and concentration of whole cells in the final beads. A range of 0.005 to 250 mg/L copper (Cu^{2+}) solutions was used to spike soil. This corresponds to copper concentrations of 0.005 to 250 mg/kg soil. The spiked soil was used in a duplicate assay setup to assess the performance of the Cu^{2+} sensor. Following the optimized assay protocol, polyacrylate-alginate-encapsulated *Bacillus subtilis* biosensor beads were incubated in copper standard solution (Figure 4a) and copper spiked clean loam soil (Figure 4b) for 5 h without shaking and then removed, washed, and their fluorescence was measured using a microtiter plate reader. Calibration curves using these data were obtained (Figure 4c). The limit of detection (LoD) of the encapsulated beads was calculated to be 0.27 mg/L and 1.26 mg/kg of Cu^{2+} for copper standard solution and copper spiked soil, respectively.

We also evaluated the performance of the encapsulated whole cell biosensor in the presence of potentially interfering metals by using spiked soil containing cobalt (7.2 mg/kg), nickel (13 mg/kg), copper (30 mg/kg), copper (50 mg/kg), and a mixture of copper (50 mg/kg), cobalt (7.2 mg/kg), and nickel (13 mg/kg), respectively (Figure 4d). In figure 4d, it can be seen that the cobalt and nickel ions, when tested at their average concentrations in soil, have minimal interference with the biosensor beads BSD8. The effectiveness of the biosensor was compared using different copper spiked soils, i.e., clean loam soil, clean clay soil, metals in soil, Everglades clay soil, and all-purpose garden soil (Figure 4e). Both clean loam and clay soil were trace metal-free or their metal concentration was negligible; and the concentration of metals in the “metals in soil” was reported in the certificate of analysis (CoA) obtained from the Sigma Aldrich website. The concentration of copper, cobalt, nickel in the soil were 188±5, 195±5, and 322±5 mg/kg, respectively, in metals in soil. The concentration of metals in the Everglades clay soil and all-purpose soil was unknown. An amount of 1.0 g of soil from each category was spiked with either 1.0 mL of 50 mg/L copper, 100 mg/L copper, or deionized water and subsequently lyophilized, before being used for this comparative assay. It should be noted that when copper (II) ions are spiked into commercially available soils that are typically used for analytical reference materials, such as the clean loam, clean clay, and metals in soils that were employed in this study, most of the ions remain bioavailable given the lack of organic content and chelators within them. However, when similar concentrations of copper (II) ions are spiked into natural untreated soils, such as the everglades clay and vigoro garden soils, some loss of bioavailability is expected due to the presence of organic content and chelators in these soils. Consequently, this loss of bioavailability was translated to the lower change in signal between commercial treated soils and natural untreated soils. Nevertheless, regardless of the soil used for spiking, the fluorescence intensity is significantly different when the soil is spiked with 50 or 100 mg/kg of copper (Figure 4f).

One of the goals of this study was to develop a portable, field deployable biosensor system. For that, the fluorescence images of encapsulated bacteria after the spiked soil assay were captured using a portable imager (Figure 5a-c). The obtained images were processed, and the light intensity of the individual polyacrylate-alginate beads was measured using ImageJ software. Calibration curves of PAA biosensor beads encapsulated biosensors were obtained by using the plate reader data and ImageJ data (Figure 5d and 5e). A Pearson correlation analysis found a strong correlation between the data obtained using the plate reader and ImageJ, with r=0.99 and p=<0.0001 for liquid assay and r=0.98 and p=<0.0001 for spiked soil assay using encapsulated beads. Since the encapsulated biosensor is intended to be portable and deployable in the field, room-temperature stored biosensor beads have been tested using spiked soil samples at soil-relevant temperatures such as 22°C, 30°C, and 40°C. As can be seen in figure 5f, the biosensor performed robustly at these temperatures. One-way ANOVA analysis showed no significant differences in fluorescence intensity change between assayed temperatures.

The long-term storage of PAA biosensor beads was evaluated by storing the beads at room temperature and at 4 °C and periodically measuring the fluorescence emission intensity of the beads in the absence and presence (50 mg/kg) of copper (II) ions for 31 days (Supplementary Figure S3).

4. Conclusions

Insertion of the ATCUN motif into the N-terminus of a circularly permutated green fluorescent protein expressed in *Bacillus subtilis* resulted in four copper responsive whole cell biosensor systems: BSD2, BSD3, BSD7, and BSD8. A unique aspect of this approach is the incorporation of self-reporting sensing proteins, cpEGFP, into *Bacillus subtilis* which allows the fluorescent protein to be expressed and encapsulated in polyacrylate-alginate beads before the use in the field. Since biosensor beads can be embedded and extracted from soil, preparation of samples is no longer necessary. The advantages of these whole cell biosensors are their cost effectiveness, metal selectivity, and portability. Although all the variants performed robustly for *in vitro* copper (II) detection, fluorescence intensity, metal selectivity, and dynamic range were the key parameters to be considered for their in-field applications. The biosensor BSD8 showed twice the fluorescence intensity than BSD3 and BSD7, and four times that of BSD2. Biosensor variants BSD7 and BSD8 are more stable at high temperature and can perform a wider range of assays in comparison to BSD2 and BSD3. BSD8 also showed higher specificity than the other variants. As a portable, field deployable biosensor, BSD8 was selected due to its fluorescence intensity, thermal stability, wider dynamic range, and selectivity. The detection limit of BSD8 was calculated to be 0.32 ± 0.08 mg/L of Cu^{2+} ions for the *in vitro* liquid culture assay. From the selectivity assay data in whole cell liquid assay, it was shown that biosensor BSD8 responds to high concentrations of cobalt and nickel, but when assayed at their relevant soil concentrations that response was significantly lowered. Furthermore, analysis with spiked soil using the same biosensor's PAA beads, the effects of cobalt and nickel on the percent change in the fluorescence intensity were minimal. Though the biosensor performance was not affected by soil relevant cobalt and nickel concentratios when used in spike soil, more work needs to be done to ensure that high concentrations of these metals do not interfere.

An important parameter of soil analysis for optimal plant growth is the measurement of bioavailable nutrients. While state-of-the-art techniques such as ICP-OES and AAS have the advantage of high sensitivity to total copper concentration, they are limited in their ease-of-use and ability to provide accurate measurements of bioavailable copper due to the intensive sample preparation methodologies needed for analysis. For an in-field assay for bioavailable Cu^{2+}, biosensor BSD8 was encapsulated in polyacrylate-alginate beads and was used to detect copper in spiked soil. The encapsulated BSD8 beads responded to spiked copper in various soils, i.e., clean loam and clay soil, metals in soil, Everglades clay soil, and all-purpose garden soil. In copper standard solution and copper spiked soil, the corresponding limits of detection (LoD) for encapsulated biosensor beads were 0.27 mg/L and 1.26 mg/kg of Cu^{2+}. The biosensor does not require an external supply of growth media and did not exhibit significant fluorescence changes after 30 days. These beads can be stored at both room temperature and 4 °C and can be used as needed. In addition, the biosensor beads performed robustly when interrogated at soil-relevant temperatures such as 22 °C, 30 °C, and 40 °C. Our MWCB approach can be applied to measurement of bioavailable levels of other micronutrients of interest for monitoring soil health by using engineered proteins that bind specific micronutrients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Early-Concept Grant for Exploratory Research (EAGER) Signals in the Soil (SitS) of the National Science Foundation (NSF), CBET-1841419. S. Daunert and S. Deo thank the following grants from the NIH R01GM127706 and 1R01MH110415-01A1 and NSF-CBET-2041413. S. Daunert is also grateful to the NIH for grants R01MH104656 and UL1TR002736, and to the Miller School of Medicine of the University of Miami for the Lucille P. Markey Chair of Biochemistry and Molecular Biology. S. Daunert and S. Deo are grateful for support from a grant from the National Institutes of Health NIGMS (Grant #R01GM114321).

References:

Adrees M, Ali S, Rizwan M, Ibrahim M, Abbas F, Farid M, Zia-ur-Rehman M, Irshad MK, Bharwana SA, 2015. Environmental Science and Pollution Research 22(11), 8148–8162.

Alloway BJ, 2013. Heavy metals and metalloids as micronutrients for plants and animals.Heavy metals in soils, pp. 195–209. Springer.

Arenas-Lago D, Andrade M, Lago-Vila M, Rodríguez-Seijo A, Vega F, 2014. Geoderma 230, 108–118.

ATSDR, 2004. Toxicological profile for Copper. Agency for Toxic Substances and Disease Registry.

ATSDR, 2005. Toxicological profile for Nickel. Agency for Toxic Substances and Disease Registry.

ATSDR, 2022. Toxicological Profile for Copper (Draft for Public Comment). Agency for Toxic Substances and Disease Registry.

ATSDR, 2023. Toxicological Profile for Cobalt (Draft for Public Comment). Agency for Toxic Substances and Disease Registry.

Ballabio C, Panagos P, Lugato E, Huang J-H, Orgiazzi A, Jones A, Fernández-Ugalde O, Borrelli P, Montanarella L, 2018. Science of the Total Environment 636, 282–298.

Chaignon V, Sanchez-Neira I, Herrmann P, Jaillard B, Hinsinger P, 2003. Environmental pollution 123(2), 229–238.

Chen P-H, Lin C, Guo K-H, Yeh Y-C, 2017. RSC advances 7(47), 29302–29305.

Choi Y-A, Keem JO, Kim CY, Yoon HR, Do Heo W, Chung BH, Jung Y, 2015. Chemical science 6(2), 1301–1307.

Cohu CM, Pilon M, 2010. Cell biology of copper. Cell biology of metals and nutrients, pp. 55–74. Springer.

Costello A, Parker J, Clynes M, Murphy R, 2020. Metallomics 12(11), 1729–1734.

Date A, Pasini P, Sangal A, Daunert S, 2010. Analytical Chemistry 82(14), 6098–6103.

Fageria N, Baligar V, Clark R, 2002. Advances in agronomy 77, 185–268.

FAO, IFAD, UNICEF, WFP, WHO, 2021. #240 p.

Fernández FG, Hoeft RG, 2009. Illinois agronomy handbook 24, 91–112.

Forzani ES, Zhang H, Chen W, Tao N, 2005. Environmental science & technology 39(5), 1257–1262.

Fraczyk T, 2021. Inorganic Chemistry 60(12), 8447–8450.

Frączyk T, 2021. Chemistry & Biodiversity 18(4), e2100043.

García-Marco S, Abadín J, Couto-Vázquez A, Martín À, González-Prieto SJ, 2020. Journal of Plant Nutrition and Soil Science 183(6), 705–717.

Graham RD, Welch RM, Bouis HE, 2001.

Harwood CR, 1992. Trends in biotechnology 10, 247–256.

Henckens M, Worrell E, 2020. Journal of Cleaner Production 264, 121460.

Hollander AK, 2018. Environmental impacts of genetically engineered microbial and viral biocontrol agents. Biotechnology for biological control of pests and vectors, pp. 251–266. CRC Press.

Inesi G, 2017. IUBMB life 69(4), 211–217.

Jung J, Maeda M, Chang A, Bhandari M, Ashapure A, Landivar-Bowles J, 2021. Current Opinion in Biotechnology 70, 15–22.

Kang Y, Lee W, Kim S, Jang G, Kim B-G, Yoon Y, 2018. Applied microbiology and biotechnology 102, 1513–1521.

Karlen DL, Veum KS, Sudduth KA, Obrycki JF, Nunes MR, 2019. Soil and Tillage Research 195, 104365.

Kim R-Y, Yoon J-K, Kim T-S, Yang JE, Owens G, Kim K-R, 2015. Environmental geochemistry and health 37(6), 1041–1061.

Kostyuk AI, Demidovich AD, Kotova DA, Belousov VV, Bilan DS, 2019. International journal of molecular sciences 20(17), 4200.

Kumar V, Pandita S, Sidhu GPS, Sharma A, Khanna K, Kaur P, Bali AS, Setia R, 2021. Chemosphere 262, 127810.

Lee JW, Chan CT, Slomovic S, Collins JJ, 2018. Nature chemical biology 14(6), 530–537.

Lehmann J, Bossio DA, Kögel-Knabner I, Rillig MC, 2020. Nature Reviews Earth & Environment 1(10), 544–553.

Li P-S, Peng Z-W, Su J, Tao H-C, 2014. Biotechnology letters 36, 761–766.

Luisi B, Hegab R, Person C, Seo K, Gleason J, 2022. ACS sensors 7(9), 2589–2596.

Mahapatra S, Yadav R, Ramakrishna W, 2022. Journal of Applied Microbiology 132(5), 3543–3562.

Maiti BK, Govil N, Kundu T, Moura JJ, 2020. Iscience 23(12), 101792.

Moraskie M, Roshid H, O'Connor G, Dikici E, Zingg J-M, Deo S, Daunert S, 2021. Biosensors and Bioelectronics, 113359.

Moya-Ramírez I, Kotidis P, Marbiah M, Kim J, Kontoravdi C, Polizzi K, 2022. ACS Synthetic Biology 11(3), 1303–1312.

Nogueirol RC, Alleoni LRF, Nachtigall GR, de Melo GW, 2010. Journal of Hazardous Materials 181(1–3), 931–937.

Pessarakli M, Haghighi M, Sheibanirad A, 2015. Adv. Plants Agric. Res 2(6), 73.

Provenzano MR, El Bilali H, Simeone V, Baser N, Mondelli D, Cesari G, 2010. Food Chemistry 122(4), 1338–1343.

Rehman M, Liu L, Wang Q, Saleem MH, Bashir S, Ullah S, Peng D, 2019. Environmental science and pollution research 26(18), 18003–18016.

Sadowski A, Baer-Nawrocka A, 2018. Land Use Policy 71, 578–583.

Sankararamakrishnan R, Verma S, Kumar S, 2005. PROTEINS: Structure, Function, and Bioinformatics 58(1), 211–221.

Sattari SZ, van Ittersum MK, Bouwman AF, Smit A, Janssen BH, 2014. Field Crops Research 157, 35–46.

Schwab S, Shearer J, Conklin SE, Alies B, Haas KL, 2016. Journal of inorganic biochemistry 158, 70–76.

Snow AA, Andow DA, Gepts P, Hallerman EM, Power A, Tiedje JM, Wolfenbarger L, 2005. Ecological Applications 15(2), 377–404.

Synhaivska O, Mermoud Y, Baghernejad M, Alshanski I, Hurevich M, Yitzchaik S, Wipf M, Calame M, 2019. Sensors 19(18), 4022.

Tang T-C, Tham E, Liu X, Yehl K, Rovner AJ, Yuk H, de la Fuente-Nunez C, Isaacs FJ, Zhao X, Lu TK, 2021. Nature Chemical Biology 17(6), 724–731.

WPP, 2019. United Nations, Department of Economic and Social Affairs, Population Division. World Population Prospects 2019: Highlights (ST/ESA/SER. A/423).

Yang W, Chow E, Willett GD, Hibbert DB, Gooding JJ, 2003. Analyst 128(6), 712–718.

Highlights:

- A sensing-reporting fluorescent protein based microbial whole cell biosensor (MWCB) for copper (II) ions was developed.
- In combination with a portable imaging system, polyacrylate-alginate encapsulated biosensor (BSD8) beads were able to measure bioavailable copper in spiked soil samples.
- Calibration curves using from ImageJ and a microtiter plate reader were generated and compared.
- Biosensor beads were stable for over a month when stored at room temperature and at 4 °C and could maintain their fluorescence and ability to detect copper throughout that time period.

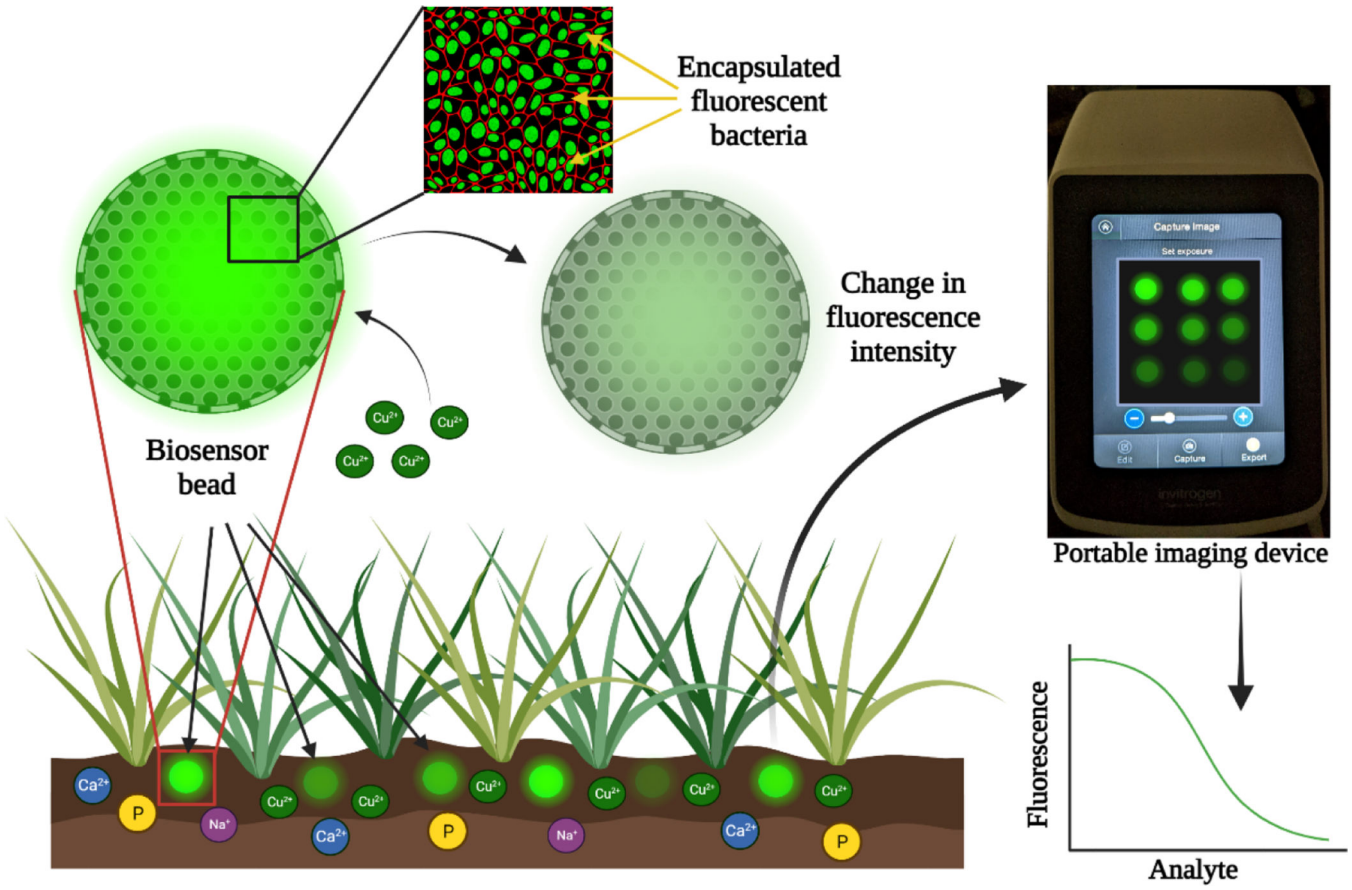

Figure 1.
Field deployable biosensing beads for the detection of bioavailable copper using a portable imaging device.

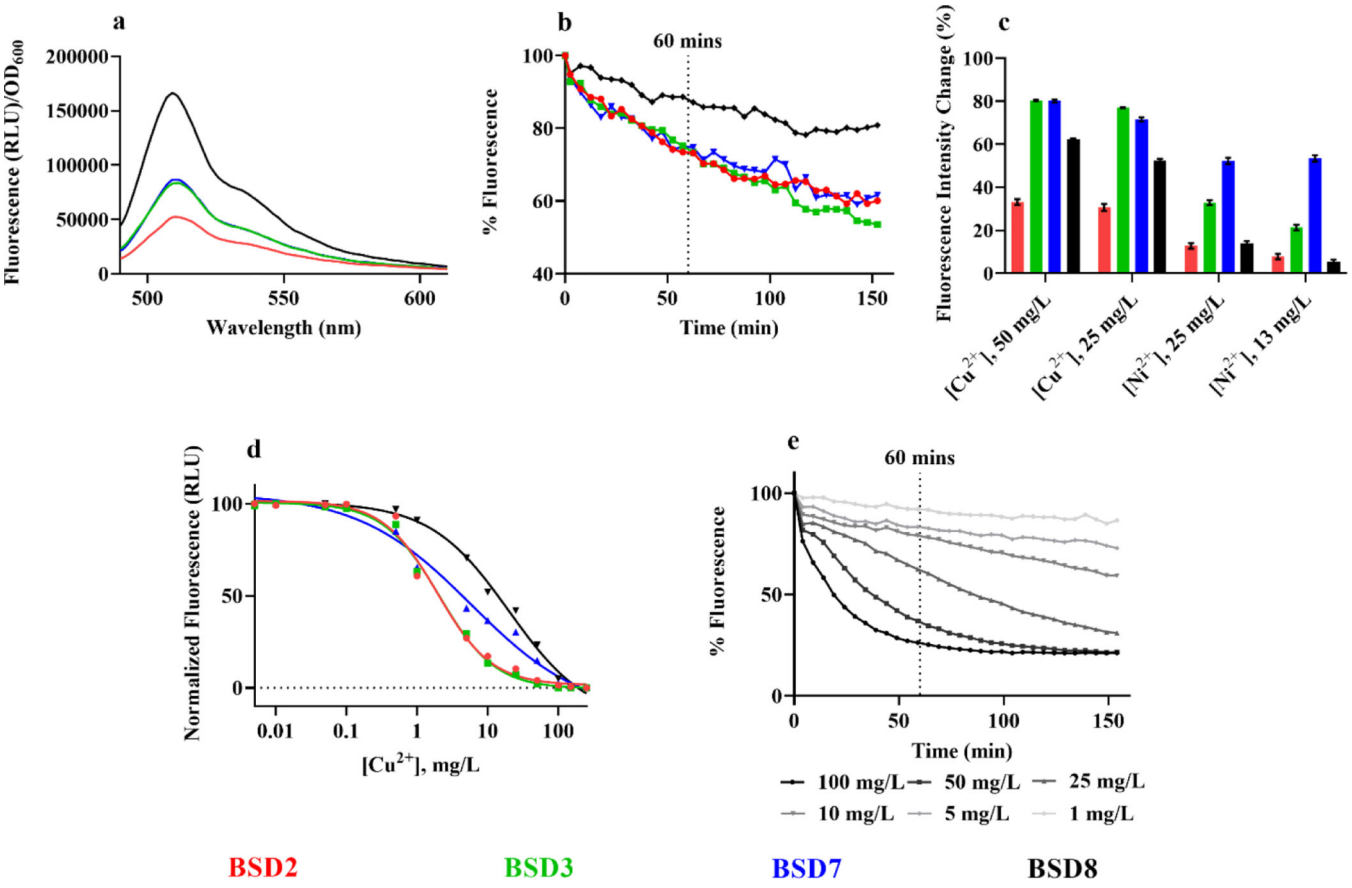

Figure 2.

Screening of microbial whole cell biosensor (MWCB) for copper (II) detection. a) Fluorescence emission spectrum scan (excitation at 462–16 nm); b) Fluorescence detection of BSD2/3/7/8 using 1 mg/L of copper (II) standard; c) Response comparison using soil relevant total Cu^{2+} and Ni^{2+} standard solution in liquid culture at 60 min assay time; d) Calibration curves of Cu^{2+} standard using *Bacillus subtilis* biosensing systems: BSD2, BSD3, BSD7, and BSD8; and e) Fluorescence intensity change with BSD8 using various concentration of copper standards.

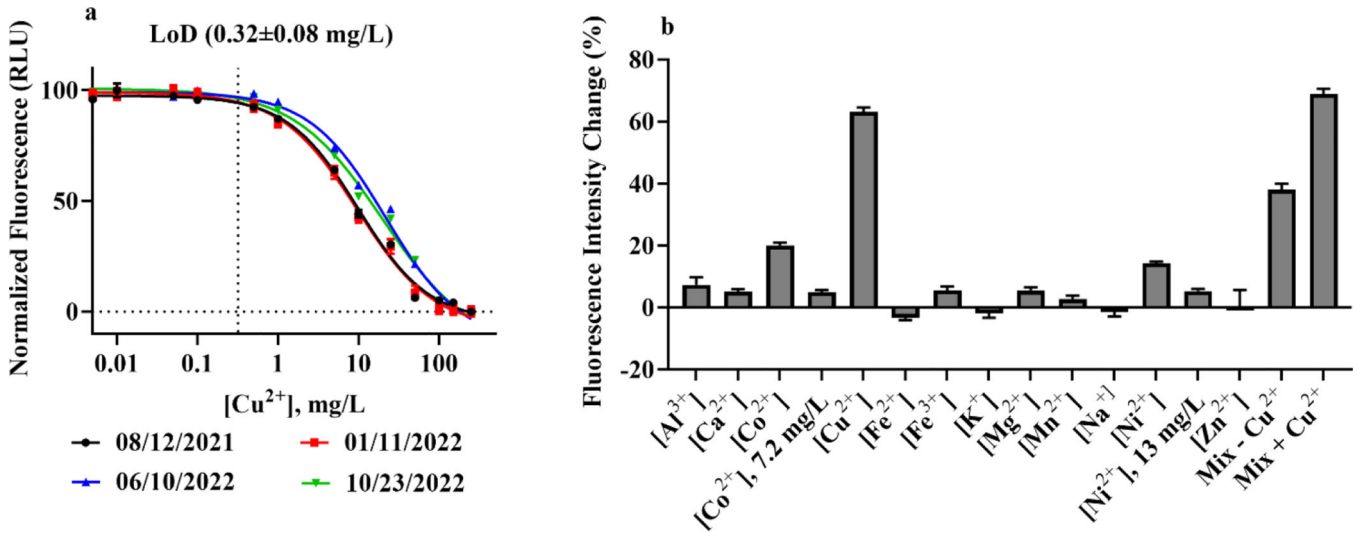

Figure 3.

Characterization of *Bacillus subtilis* biosensor system BSD8 in liquid assay. a) Calibration curve of whole cell biosensor system BSD8 using Cu^{2+}standards at different days; and b) Metal selectivity studies of BSD8 at 50 mg/L of listed metals unless specified.

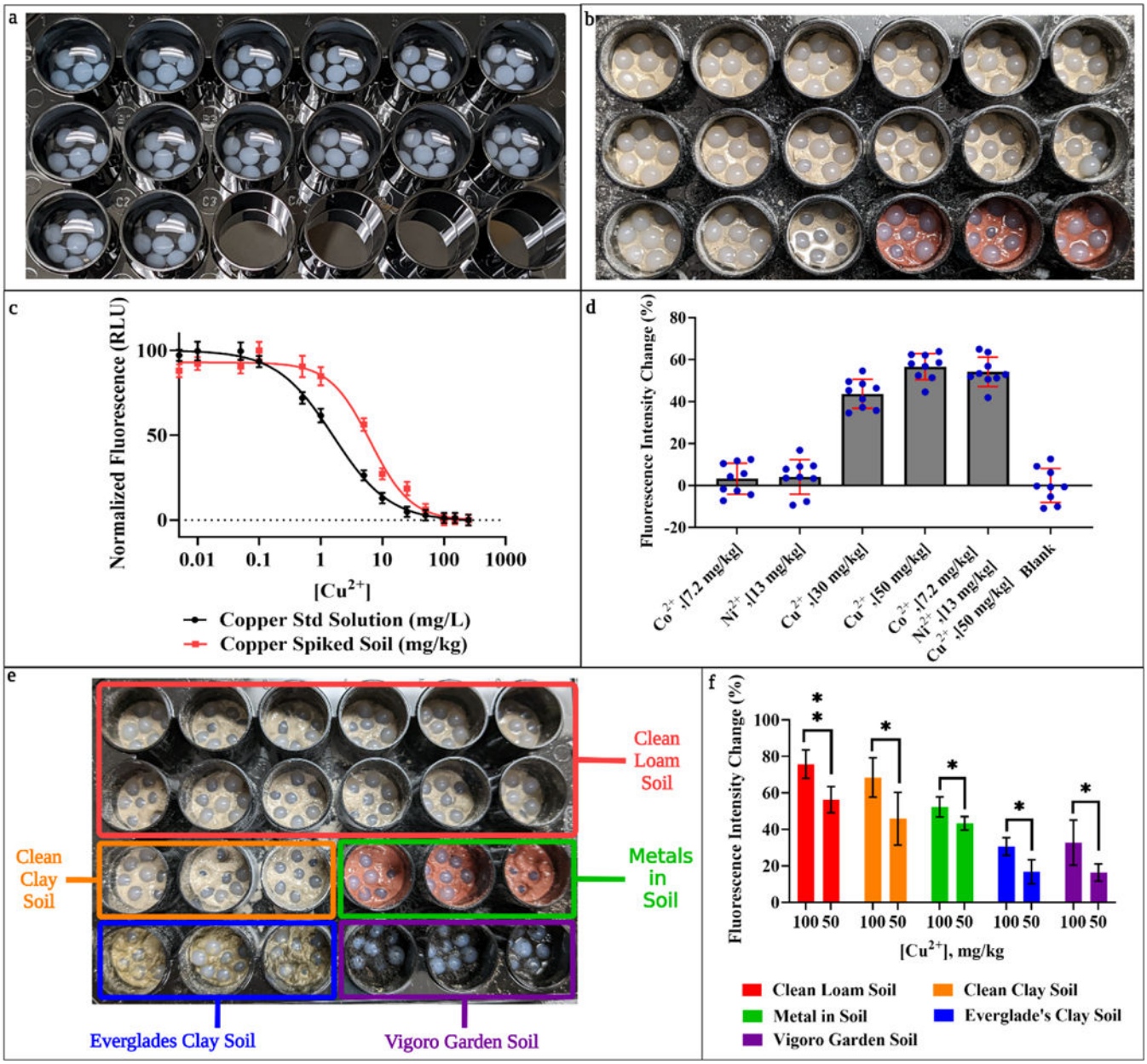

Figure 4.

Characterization of polyacrylate-alginate encapsulated MWCB beads. Image of encapsulated polyacrylate-alginate beads for (a) copper standard solution assay and (b) copper spiked clean loam soil assay; c) Calibration curve using the microtiter plate reader data of extracted beads from liquid and spiked soil assay; d) Metal selectivity assay in soil spiked with cobalt, nickel, copper, and a mixture of a mixture of copper, cobalt and nickel (microtiter plate reader data); e) Assay plate layout with different soils spiked with copper and BSD8 alginate beads; and f) Fluorescence intensity of the BSD8 biosensor in different soils spiked with 50 and 100 mg/kg of copper.

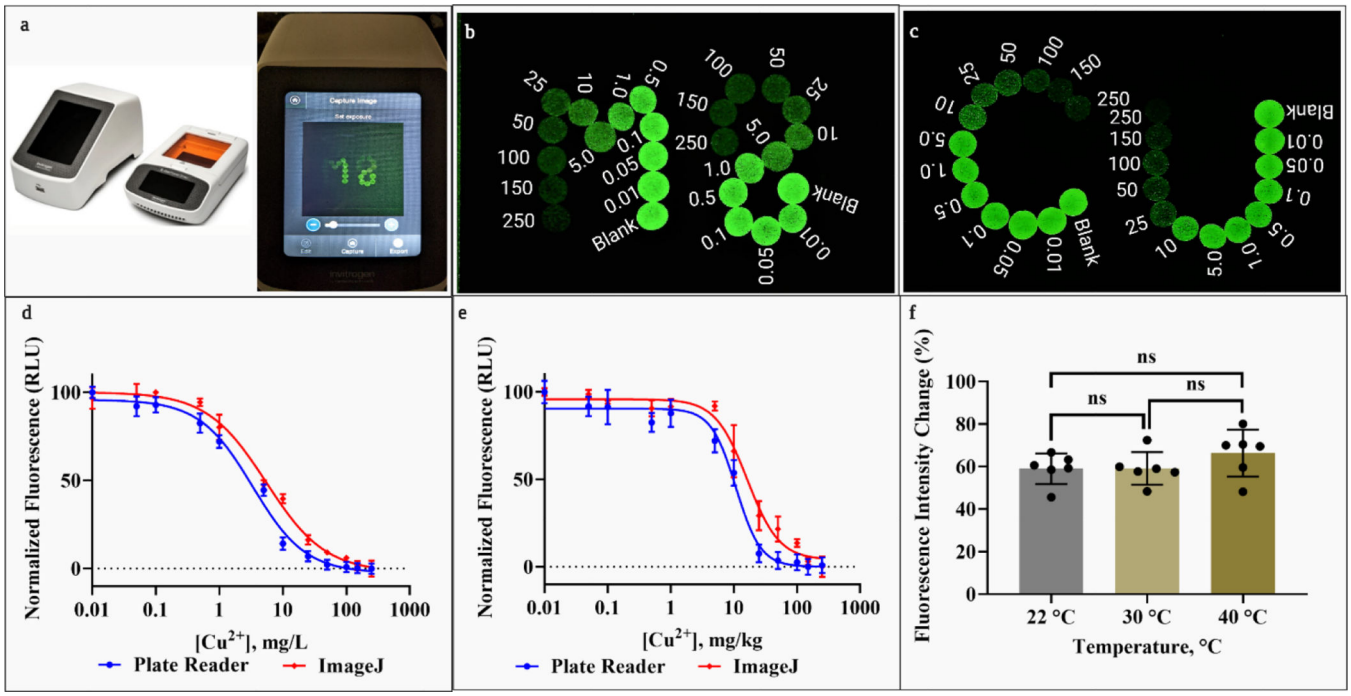

Figure 5.

Instrumentation for the field-deployable portable copper biosensing system. a) Invitrogen E-Gel Power Snap instrument and fluorescent image of BSD8 alginate beads; Images of alginate beads containing whole cell biosensor system of BSD8 after 5.0 h of incubation into (b) copper standard solution and (c) copper-spiked clean loam soil; Calibration curve of extracted beads from (d) copper standard solution and (e) copper-spiked soil using the plate reader and ImageJ data. A strong positive Pearson correlation was found between the plate reader and ImageJ data for both copper standard solution and copper-spiked soil assay using alginate beads with r=0.98 and p<0.0001. f) Soil spiking assay using BSD8 biosensor beads at soil relevant temperatures. Clean loam soil was spiked using 50 mg/kg of copper standard and deionized water as a blank. One way ANOVA analysis showed no significant differences in fluorescence intensity change between different incubation temperatures.